REQUIREMENT OF IRON FOR PLATELET PROTEIN SYNTHESIS

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SUMMARY: 2,2'-bipyridine, a potent chelator of iron, reduced platelet protein synthesis by 80%. This could be prevented by addition of either transferrin at 5 μM or hemin at 0.3 to 0.8 mM. Transferrin alone activated protein synthesis by 48% in 18 out of 28 experiments, whereas iron salts had no effect. Hemin alone activated protein synthesis in all experiments by as high as 170%. The hemin effect was different from the transferrin effect in that hemin activation was associated with platelet lysis and decreased platelet protein recovery. Never-the-less the specific activity obtained with hemin incubations was 2.7 fold greater than the specific activity obtained from water incubations in which comparable lysis was obtained. It is concluded that iron is required for platelet protein synthesis.

Previous work from this laboratory has shown that iron is required for maximum platelet production in acute or chronically bled animals via the production of megathrombocytes (young platelets) (1,2). It was postulated that one function of iron was to act directly or indirectly as an essential compound for the synthesis or production of an integral part of the platelet. Since one of us participated in work showing that hemin (or iron for hemin synthesis) is required by reticulocytes for maximal protein (hemoglobin) synthesis (3), the present study was undertaken to investigate if hemin and iron are similarly required by platelets, in which hemoglobin is not present. This proved to be the case, indicating that the requirement of hemin or iron was not specific for hemoglobin protein synthesis. The data suggest that indeed hemin or iron may be a universal requirement for protein synthesis.

MATERIALS AND METHODS: Both control and phenylhydrazine-treated rabbits were employed for platelet protein synthesis studies. Phenylhydrazine treatment provides a thrombocytosis with an increased number of megathrombocytes (4). Phenylhydrazine-treated rabbits were prepared by daily subcutaneous injection with 0.25 ml/kg of 2.5 percent neutralized phenylhydrazine hydrochloride for four days. Megathrombocytes were determined by Coulter Counter (5). These were found to be rich in ribosomes. Ribosomes were also noted in control platelets, but these were fewer in number (6). Blood was withdrawn from an ear artery into a plastic syringe containing ACD-A anti-

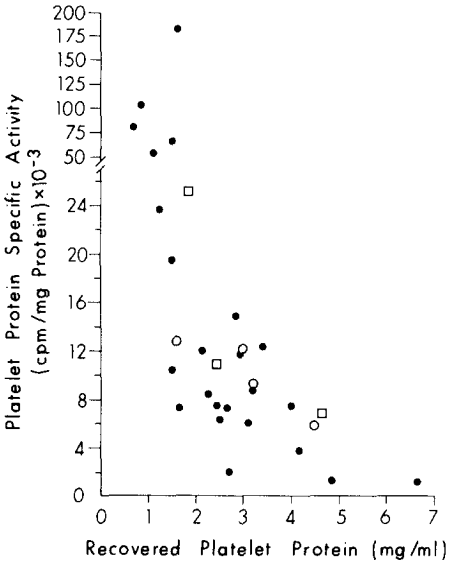

Figure 1. Correlation between platelet suspension volume (recovered protein) and platelet protein synthesis. Various platelet suspensions (1-7 volume %) were incubated in a Ringer's solution at 37° for 1 hour in the presence of a mixture of radioactive amino acids, without further additions. The platelet protein recovered was assayed for radioactivity and protein content. The ordinate refers to cpm/mg protein. The abscissa refers to recovered protein. The platelet suspension volumes were found to be directly proportional to recovered protein. Open circles and squares refer to experiments at different suspension volumes with platelets from the same animal.

coagulant (blood diluted 9:10). Platelets were collected and processed as described previously (7), washed in modified human Ringer's solution (8), and suspended in this solution to platelet volumes of 1-7 percent.

The platelet suspensions were incubated at 37° as described previously (9). After 10-20 minutes preincubation with various additives (described below), 5 μc of a mixture of [^{14}C] amino acids (Amersham-Searle, 54 mc/mA, protein acid hydrolysate) was added for one hour. Incubations were terminated by centrifugation of the incubation flasks at 2,500 g for 10 minutes at 4°. The platelets were washed twice with 1% ammonium oxalate, and once with a modified human Ringer's solution (to constant supernatant radioactivity). The platelet suspensions were then transferred to 15 ml test tubes which were centrifuged to remove the Ringer's solution. The pellet was then treated with 20 times its volume of chloroform-methanol (2:1) and held over a vortex mixer for one minute (9). The platelet homogenate was then stored overnight

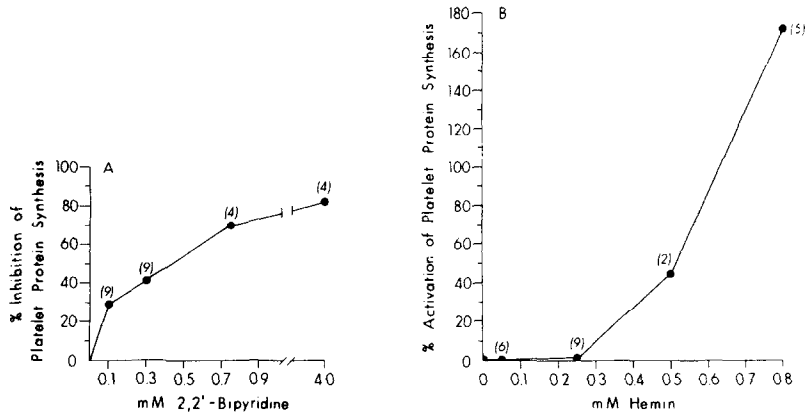

Figure 2. Effect of A. 2,2'-bipyridine chelation of iron and B. hemin on platelet protein synthesis. Number of experiments are given in parentheses. A concentration of 0.25 mM hemin was occasionally noted to activate protein synthesis (data not shown).

at 4°. It was then centrifuged to obtain the protein precipitate which was treated by the Schneider procedure (10). The precipitate was then heated for 5 minutes at 60° in ethanol:ether (3:1). The ethanol:ether was removed by centrifugation, the precipitate dried and then taken up in 1 ml of 0.5 N NaOH. Radioactivity was determined as described previously (9). Protein was determined with the biuret reagent, employing bovine serum albumin as standard. Data are expressed as cpm/mgm protein.

RESULTS AND DISCUSSION: Preliminary studies on platelet volume suspensions indicated the phenomenon of propinquity. Platelet protein synthesis was inversely proportional to platelet concentration (Figure 1). Similar observations were noted with platelet glycogen synthesis (11). At platelet suspensions of 2-3 volumes percent, protein synthesis was roughly proportional to megathrombocyte number ($r = 0.89$, $p < 0.05$, $n = 6$) confirming previous work (9) that heavy-large platelets are more active in protein synthesis. Both puromycin (200 μg/ml) and cycloheximide (10^{-2} M) were capable of almost completely inhibiting incorporation of radioactivity into platelet protein; 88% and 92% inhibition, respectively (average of four experiments), indicating specificity of protein synthesis.

The effect of iron chelation is shown in Figure 2A. Thus, 2,2'-bipyridine, a potent chelator of iron, could reduce platelet protein synthesis by 80%. This inhi-

TABLE I

RELEASE OF 2,2'-BIPYRIDINE INHIBITION OF PLATELET PROTEIN SYNTHESIS WITH TRANSFERRIN AND TRANSFERRIN + IRON

EXPERIMENT*	CONTROL	BIPYRIDINE (mM)	BIPYRIDINE + TRANSFERRIN (5μM)	BIPYRIDINE + TRANSFERRIN + IRON (2mM)
1	50,797	27,895 (0.1)	42,422	
2	11,817	7,943 (0.1)	8,952	9,528
3	12,214	5,426 (0.3)	9,699	12,644
4	14,845	3,182 (1.0)	4,021	
		1,948 (4.0)	1,830	

*A 2.5 volume per cent platelet suspension was preincubated for 10 minutes with either of the above additions at 37°. The radioactive amino acid mixture was then added for 60 minutes. Data are expressed as cpm/mg protein.

bition could be prevented with rabbit transferrin (Miles Laboratories) or transferrin plus iron, Table I. Indeed transferrin alone, at concentrations of 5 μM, activated protein synthesis by 48% in 18 out of 28 experiments. Iron alone in the form of ferrous ammonium sulfate (2 mM) had no effect on protein synthesis in three experiments.

Similar results were obtained with hemin, however these should be interpreted with caution. Thus, although hemin could overcome the inhibitory effect of iron chelation, Table II, and hemin alone could activate protein synthesis (specific activity) by 170%, Figure 2B, this was associated with significant platelet lysis (release of 280 mμ absorbing material into the incubation supernatant) and diminished protein recovery. These results could be explained by two possibilities: 1) Hemin lysed platelets which were more susceptible to lysis and less capable of synthesizing protein, i.e. older platelets compared to younger platelets (9); or in the same context, hemin lysis of platelets resulted in the selective retention of a high specific activity protein (perhaps membrane-attached). 2) Despite lysis of platelets, hemin still activated protein synthesis. Experiments were therefore designed to gain more

TABLE II

RELEASE OF 2,2'-BIPYRIDINE INHIBITION OF PLATELET PROTEIN SYNTHESIS WITH HEMIN

EXPERIMENT*	CONTROL	BIPYRIDINE (mM)	BIPYRIDINE + HEMIN (mM)
1	19,516	5,758 (1.0)	7,451 (0.6)
		6,507 (0.5)	12,688 (0.6)
2	7,197	2,743 (0.5)	3,581 (0.3)
		3,539 (0.3)	8,975 (0.3)
		3,866 (0.1)	18,140 (0.3)
3	50,797	23,154 (0.3)	46,056 (0.8)
		27,895 (0.1)	89,004 (0.8)

*Various platelet volume suspensions were preincubated with the above additions for 10 minutes at 37°. The radioactive amino acid mixture was then added for 60 minutes. Data are expressed as cpm/mg protein.

information concerning the mechanism of the hemin effect. Platelets were incubated in varying concentrations of distilled water:human Ringer's solution mixtures and the specific activity determined. When comparable protein recoveries were compared with hemin experiments, a 2.7 fold greater specific activity was still obtained with hemin in 2 separate experiments. Additional experiments were performed with carrier protein (5 mg/ml albumin added just prior to the chloroform:methanol step in order to minimize radioactive protein loss). The effect of hemin on the inhibitory action of 2,2'-bipyridine was examined by measuring total cpm only. In 5 out of 5 experiments total counts were restored to normal in hemin plus 2,2'-bipyridine incubations in contrast to 2,2'-bipyridine incubations alone. Thus the average of 5 control experiments was 22,206 cpm compared to 16,098 cpm with 0.1 mM 2,2'-bipyridine alone and 23,556 cpm with 0.1 mM 2,2'-bipyridine plus 0.8 mm hemin. These data suggest that hemin may be activating residual protein synthesis, despite platelet lysis.

In evaluating our data, one should consider the possibility that incorporation of radioactive amino acids into protein was due to contamination by either leukocytes or reticulocytes. We consider this unlikely, because the ratio of these cells to platelets was approximately 1:1000 for each. Furthermore, more than half the experiments were performed with control rabbits, where reticulocyte contamination could not be introduced as a variable.

These studies present evidence that the platelet requires iron for maximum protein synthesis. The demonstration that transferrin and possibly hemin prevent the inhibition of protein synthesis by iron deficiency suggests that iron in the form of transferrin or hemin may be utilized by the platelet to synthesize protein. This requirement raises the possibility that iron in the form of hemin might be used for initiation of protein synthesis in platelets in a similar fashion as has been shown for hemoglobin synthesis in intact reticulocytes (3,12-13) and reticulocyte cell-free systems (3,14-19). Recently, while our manuscript was in preparation, a report was published describing an intact as well as cell-free system from Krebs ascites tumor cells in which hemin was required for maximum protein synthesis (21). It is possible, therefore, that all mammalian cells require hemin or transferrin for initiation of protein synthesis. While this view is still speculative, it is clear that iron plays an important role in the control of platelet protein synthesis. Indeed, mitochondria, an active Krebs cycle and heme-containing enzymes have been demonstrated in platelets (22).

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